

Susceptibility to cisplatin-induced hearing loss in CHOP knockout mice

Honors Undergraduate Research Thesis

Presented in Partial Fulfillment of the Requirements for graduation “with Honors Research
Distinction in Speech and Hearing Science” in the undergraduate colleges of The Ohio State

University

by

Izabela Jamsek

The Ohio State University

April 2016

Project Advisor: Dr. Eric Bielefeld, Department of Speech and Hearing Science

Abstract

Cisplatin is a chemotherapeutic drug that causes hearing loss, and the mechanisms of its ototoxicity have been under study for several years. A key mechanism of cisplatin ototoxicity is apoptosis of the sensory hair cells. The cellular signals that trigger cisplatin-induced apoptosis in the cochlea are still unclear. The current experiment tested the hypothesis that endoplasmic reticulum stress and the unfolded protein response (UPR) are triggers for cochlear apoptosis. C/EBP homologous protein (CHOP) is a key signaling molecule in UPR-mediated apoptosis. Therefore, we tested susceptibility to cisplatin-induced hearing loss in CHOP knockout mice. Removing the CHOP gene was hypothesized to prevent cisplatin-induced cellular apoptosis and subsequent hearing loss. Electrophysiological hearing thresholds obtained through auditory brainstem response tests were used to assess hearing in CHOP knockout mice and C57Bl6/J controls. The mice were then exposed to cisplatin at four doses (8, 10, 14, 16 mg/kg) administered through intra-peritoneal injections. Post-exposure thresholds were obtained on Day 3 and Day 7 after cisplatin. An initial discovery in the study was that the CHOP knockout mice did not have equivalent hearing sensitivity to the controls. They had higher baseline thresholds, indicating initial hearing loss likely due to developmental damage/malformation of the cells in the cochlea. The finding implies that the UPR is crucial for the normal development of the auditory system. The major finding from the study was less cisplatin-induced hearing loss in the CHOP knockout mice. At each drug dosage, the mean threshold shift after cisplatin was higher in the control group than in the CHOP knockout group. This supports the hypothesis that CHOP is a potential triggering molecule for cisplatin-induced apoptosis in the cochlea. The finding could lead to new methods for preventing cisplatin ototoxicity, and future studies will attempt to modulate the UPR as one of these methods.

1. Introduction

Cisplatin is a platinum-based drug frequently used for chemotherapy treatments in human patients due to its effectiveness at fighting many different types and stages of cancers, including testicular, ovarian, bladder, and cervical (Rybak & Ramkumar, 2007). Its widespread use and effectiveness create especial concern for its damaging side effects that include ototoxicity.

Ototoxicity is defined as the functional impairment of the hearing mechanism by a drug or agent, and is usually caused by cellular destruction in the cochlea (Rybak & Ramkumar, 2007). Unlike other sensory systems (Sammata & McClintock 2010), the cochlea does not regenerate sensory transduction cells that have died, resulting in the permanent nature of most sensorineural hearing losses (Yost, 2008). The permanence of cisplatin ototoxicity makes its prevention imperative.

The proportion of people who develop hearing loss due to cisplatin has been documented at rates as high as 90% of patients who received the minimum experimental dose (Hayes et al. 1977). Cisplatin damages inner hair cells and supporting cells of the cochlea, but most significantly affects the cochlear outer hair cells beginning at the base of the cochlea (Ding et al, 2006). The apoptotic lesion spreads throughout the cochlea toward the apex as cisplatin dose and duration increase (Fleischman et al, 1975). The basal outer hair cells initially affected correspond to a high-frequency hearing loss (6 kHz and above in the human) that progresses into the middle frequencies (1.5-6 kHz) as cisplatin treatment continues (Madasu et al., 1997). High-frequency hearing corresponds perceptually with speech recognition. Difficulty with speech recognition due to a high-frequency hearing loss typical for initial cisplatin treatment can have a negative impact on the quality of a patient's daily communication, and especially impairs the pediatric population's language acquisition (Knight, Kraemer, & Neuwelt 2005).

The permanence of sensorineural hearing loss and the communicative and developmental issues imposed by cisplatin-induced hearing loss are side effects of a high risk chemotherapy treatment implemented to save patient lives. As cisplatin application continues to become more effective, the need to prevent the high incidence of the side effect of permanent hearing loss shifts to the focus of exploratory research to improve patient quality of life. The biggest issue facing such research is that cisplatin's ototoxicity is caused by many of the same mechanisms that produce its chemotherapeutic effects. Consequently, protection from cisplatin ototoxicity requires theoretical and clinical applications that reduce or eliminate the damaging ototoxic pathways of the drug while retaining its chemotherapeutic qualities (Hayes et al, 1977; Rybak & Ramkumar 2007).

Cisplatin's initial course in the cochlea after administration begins in cell nuclei, where its platinum atom covalently binds to cell DNA. The subsequent downstream effects cause DNA damage and activate various apoptosis pathways in the cochlea, resulting in ototoxicity (Rybak & Ramkumar, 2007). Therefore, the current project is part of an ongoing line of research to identify apoptosis triggers and signaling pathways in cisplatin-induced hearing loss. If cellular pathways of cisplatin-induced apoptosis are identified, cochlear protection strategies for cisplatin treatment could be developed and administered to patients to partially or completely prevent hearing loss.

Prolonged endoplasmic reticulum (ER) stress and activation of the unfolded protein response (UPR) are hypothesized to participate in the pathway of cochlear apoptosis from cisplatin exposure due to its general downstream apoptosis effects in sensory cells (Sammata & McClintock 2010). The ER's primary function in the cell is to fold and activate proteins for transport. This process can be interrupted and stressed in a number of ways, including ischemia,

hypoxia, heat shock, amino acid starvation, and gene mutation, in which case the ER undergoes a series of gene expressions that either result in cell survival or apoptosis (Fujinami et al, 2012).

The UPR is one of the pathways activated to prevent the accumulation of irregular and ineffective proteins that are the ER's output under stress. When the ER is stressed beyond the UPR's capacity to restore its function, the UPR triggers cellular apoptosis pathways. UPR-mediated apoptosis pathways are active in other sensory cell injuries (Sammata & McClintock, 2010) and in a model of age-related cochlear injury (Wang et al. 2015). One of the proteins identified in UPR's apoptosis pathway, CCAAT/-enhancer-binding protein homologous protein (CHOP), promotes sensory cell apoptosis when activated (See Figure 1) (Yan et al, 2015).

In this project, CHOP knockout mice were hypothesized to have reduced susceptibility to cisplatin-induced hearing loss. The hypothesis is based on a model of cisplatin-induced hearing loss in which cisplatin induces ER stress, thus activating the UPR. With high doses of cisplatin that cause hearing loss, the ER stress triggers UPR-mediated apoptosis involving its downstream signals, including CHOP. Therefore, the knockout of CHOP will interrupt the apoptosis signaling sequence, and subsequently reduce susceptibility to apoptosis in the cochlea. Long-term, identifying the cell death pathways that cisplatin triggers in the cochlea could also illuminate the similarities and differences between cisplatin- and noise-induced hearing loss pathways with further research, and allow for the development of treatments to protect against those forms of hearing loss.

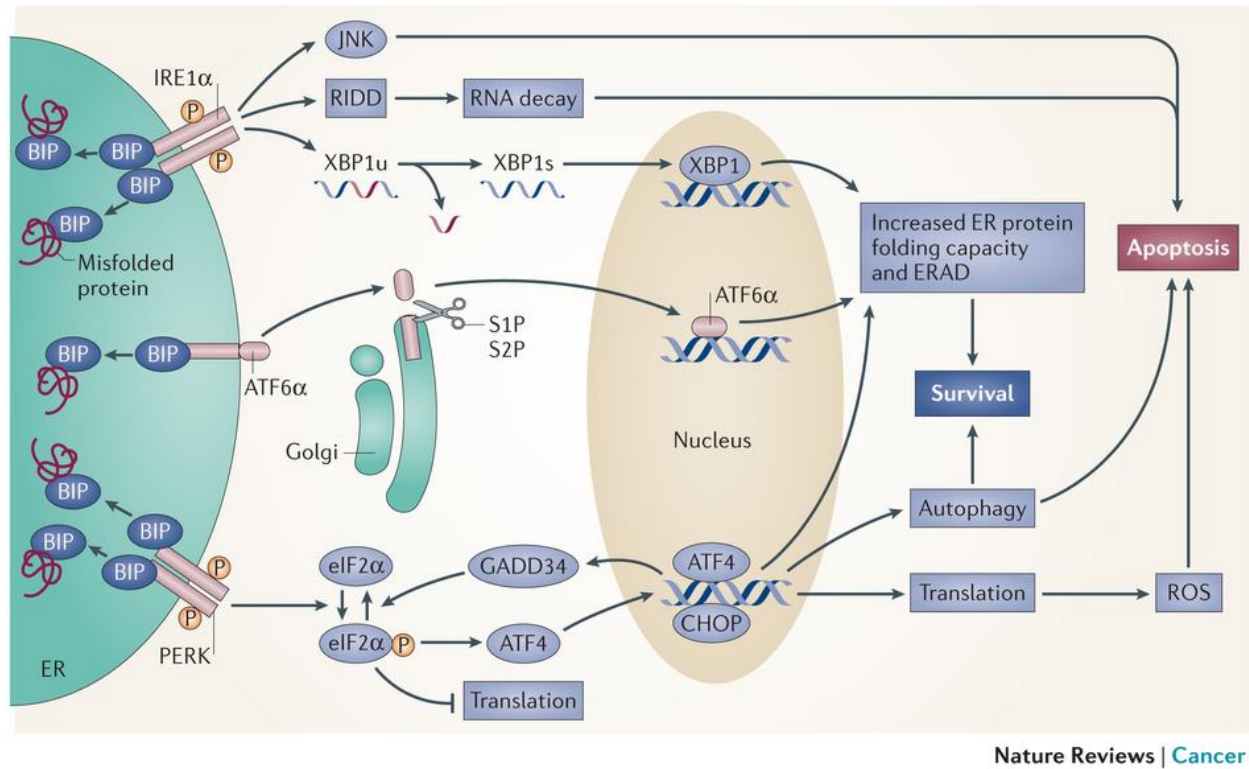


Figure 1. Schematic showing UPR apoptosis pathways, including CHOP (Erguler et al., 2013).

Expected Results

The expected results from this research were that cisplatin would induce a smaller amount of threshold shift at each dose in the CHOP knockout mice than the control C57Bl6/J mice. Additionally, we expected that successive increases in cisplatin dosage would result in more hearing loss within all groups. As cisplatin increased in the auditory system, the amount of stress on the ER would increase, increasing its apoptotic response. While the effect would be lessened in the CHOP knockout mice, it was still expected that they would show hearing loss from cisplatin, as there are multiple other mechanisms through which cisplatin could induce injury in the cochlea.

2. Methods

Subjects

Adult (at least four weeks of age) C57Bl6/J mice were used in the study: 24 in the control group and 27 in the CHOP knockout group, for a total of 51 mice for the study. All of the C57Bl6/J mice and six breeding pairs of CHOP knockout mice were acquired from Jackson Laboratories (Farmington, CT). All mice were kept in a quiet colony (<45 dB Leq for any eight-hour period). Offspring of CHOP knockout breeders were weaned at 21 days of age. No breeding pair generated more than five litters of offspring. All proceedings involving the animals were approved by The Ohio State University's Institutional Animal Care and Use Committee.

Auditory Brainstem Response (ABR) Testing

Baseline hearing thresholds were obtained at three to four weeks of age using auditory brainstem responses (ABR). In order to perform ABR tests, the animals were anesthetized with a mixture of gaseous isoflurane and oxygen. For initial anesthetization, 2.5% isoflurane was used with a 1 L/min O₂ flow rate. Following anesthesia, the animals were placed in a sound booth with a nose cone supplying a mixture of 1.2% isoflurane and oxygen for the duration of the test. In the sound booth, three platinum electrodes were inserted subcutaneously, one in the vertex, one behind the right pinna, and the ground near the left rear leg of the animal. All acoustic stimuli for the ABRs were generated using Tucker Davis Technologies (TDT, Gainesville, FL) SigGen software. Each stimulus was a tone burst that was 1 ms in duration, and had a 0.5 ms rise and fall time with no plateau. Stimuli were presented at a rate of 19/sec. Signals were routed to a speaker (TDT Model MF1) positioned at either 90 or 270 degrees azimuth (either directly next to the right or left ear), 6 cm from the vertex of each rat's head. One ear, randomly-selected, was tested in each animal. Acoustic stimuli were calibrated prior to each testing session, by recording

the output of the speaker with a microphone placed at the animals' head level. The evoked responses were amplified with a gain of 50,000, using a TDT RA4LI headstage connected to a TDT RA4PA pre-amplifier, and bandpass filtered from 100-3000 Hz. Four hundred sweeps were averaged at each stimulus level using TDT BioSigRz software. Six frequencies were tested: 4, 8, 12, 16, 24, and 32 kHz, at decreasing 5 dB intervals from 90 dB SPL to 20 dB SPL. If threshold was reached before 20 dB SPL, levels below threshold were skipped to reduce testing time.

The human ABR consists of the first five waves of electrical activity post-stimulus. These five waves reflect the activity of the structures of the central auditory system as the signal progresses from the cochlea to the primary auditory cortex and they occur within 10 ms of exposure to a noise stimulus (Yost, 2008). In the mouse, the ABR is dominated by two positive and two negative peaks. ABRs were recorded and analyzed with BioSigRZ. To determine the threshold of the animals at individual frequencies, the ABRs at each dB SPL were compared. Threshold was defined as the lowest point at which a brainstem response wave could be discerned (See an example in Figure 2).

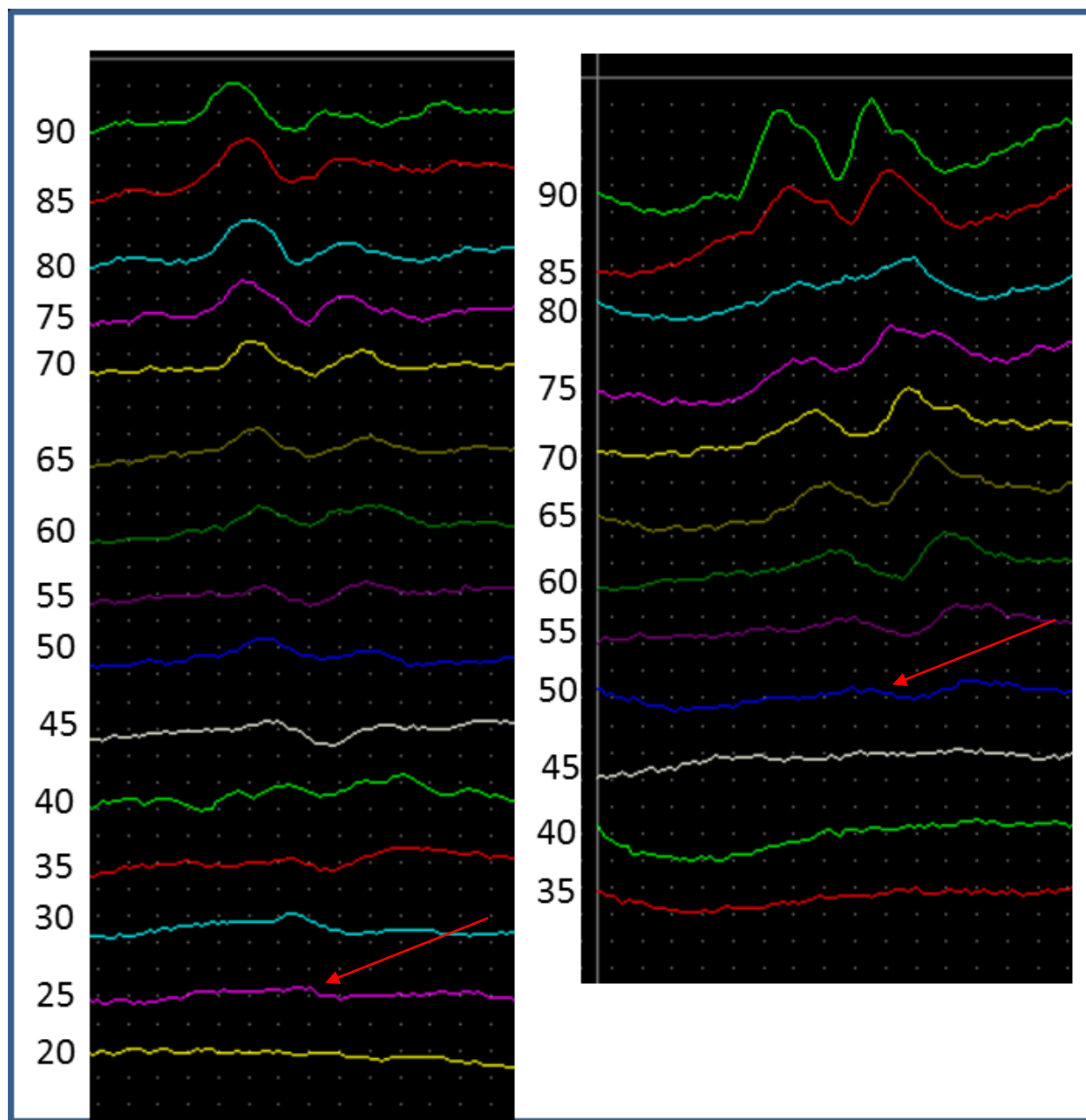


Figure 2. The worksheet view of BioSigRZ showing mouse ABR readings at 16 kHz in dB SPL. The red arrows indicate where threshold would have been measured. The first column is a pre-exposure baseline sample, and the second is a post-exposure Day 7 sample.

Experiment

The experiment was divided into three phases: pretest, cisplatin injection, and follow-up testing. The pretest phase involved an ABR test to determine baseline thresholds. Following the pretest phase, the animals were injected with a single dose of 8 (C57 n=6, CHOP -/- n=11), 10 (C57 n=6, CHOP -/- n=9), 14 (C57 n=6, CHOP -/- n=0), or 16 (C57 n=6, CHOP -/- n=7) mg/kg of cisplatin. The solution of cisplatin was made by dissolving solid cisplatin into a saline solvent and using a magnetic agitator with mild heat to facilitate the dissolution. All doses were administered via intra-peritoneal injection. The animals were tested again with ABR testing at Day 3 and Day 7 post-cisplatin injection to determine threshold shift due to cisplatin. Threshold shifts were calculated by subtracting the baseline thresholds from the post-cisplatin thresholds. In the example in Figure 2, the pre-exposure threshold was 25 dB SPL, and the post-exposure was 50 dB SPL, indicating a threshold shift of 25 dB. Cisplatin is known to induce little, if any, temporary threshold shift (TTS), so all hearing loss documented was considered permanent threshold shift (PTS).

Statistical analyses

The significance of the group differences were determined via analyses of variance (ANOVA). Two-factor ANOVAs (group x frequency) were used to delineate differences between pre-exposure thresholds for the C57B16/J controls versus the CHOP -/- mice. For the dose response of cisplatin and hearing loss in the C57B16/J mice, a three-factor ANOVA was used (dosing group x frequency x test day), with test day treated as a repeated measure. For comparison of controls versus CHOP -/- in threshold shift from cisplatin, each dose level was analyzed separately with two-factor ANOVAs (group x frequency). Frequency was treated as a between-subjects variable because of the differential effect of cisplatin on higher frequencies

compared to lower. Independent samples t-tests and Tukey A tests were used for pairwise comparisons as *post hoc* tests to determine individual group differences.

3. Results

Pre-exposure thresholds

Figure 3 displays pre-exposure thresholds for the 24 C57B16/J control mice and the 27 CHOP $-/-$ mice. The pre-exposure thresholds showed that the CHOP knockout mice did not have equivalent hearing sensitivity to the controls. On average, the CHOP group had elevated mean baseline thresholds compared with the C57B16/J group. A two-factor ANOVA (group x frequency) revealed a group x frequency two-way interaction ($p < 0.001$). T-tests were used to compare the two groups at each frequency. Significant differences, in which the CHOP $-/-$ had higher thresholds than the controls, were found at 8 ($p = 0.002$), 24 ($p = 0.004$), and 32 kHz ($p < 0.001$).

Threshold shift from different doses of cisplatin

The first experimental goal of the project was to measure a dose response in the C57B16/J controls for cisplatin-induced hearing loss. Groups of six animals each were given either 8, 10, 14, or 16 mg/kg of cisplatin and were tested three and seven days later. Results are displayed in Figures 4 and 5. A three-factor ANOVA (group x frequency x test day) was used to analyze the differences in threshold shift of the four doses. A significant two-way group x test day interaction ($p < 0.001$) was found. *Post hoc* Tukey A evaluations revealed that at Day 3, the 16 mg/kg induced more threshold shifts than the 8 or 10 mg/kg doses ($p < 0.001$). There was a statistical trend for 16 mg/kg to be greater than 14 mg/kg ($p = .057$) but it did not reach the criterion level of 0.05. At Day 7, the 16 mg/kg dose induced greater threshold shifts than each of

the other three doses ($p < 0.001$). At both Day 3 and Day 7, none of the other doses (8, 10, or 14 mg/kg) induced threshold shifts that were significantly different from one another.

Cisplatin-induced threshold shift in CHOP -/- mice

The second experimental goal was to test if the CHOP -/- mice had different susceptibility to cisplatin-induced hearing loss compared to the controls. The major finding from the study was less cisplatin-induced hearing loss in the CHOP -/- mice at Day 7 after exposure. Results for 8, 10, and 16 mg/kg are displayed in Figures 6-8, respectively. Threshold shifts between the C57B16/J and CHOP -/- were analyzed separately at each dose level with two-factor (group x frequency) ANOVAs. At each drug dosage, the mean threshold shift after cisplatin was higher in the control group than in the CHOP knockout group. At 8 mg/kg, the differences between the two groups were not significantly different at any frequency. At 10 mg/kg, there was a significant main effect of group ($p < 0.001$), indicating that the CHOP -/- threshold shifts were significantly lower than the controls. The same result was found at 16 mg/kg ($p < 0.001$).

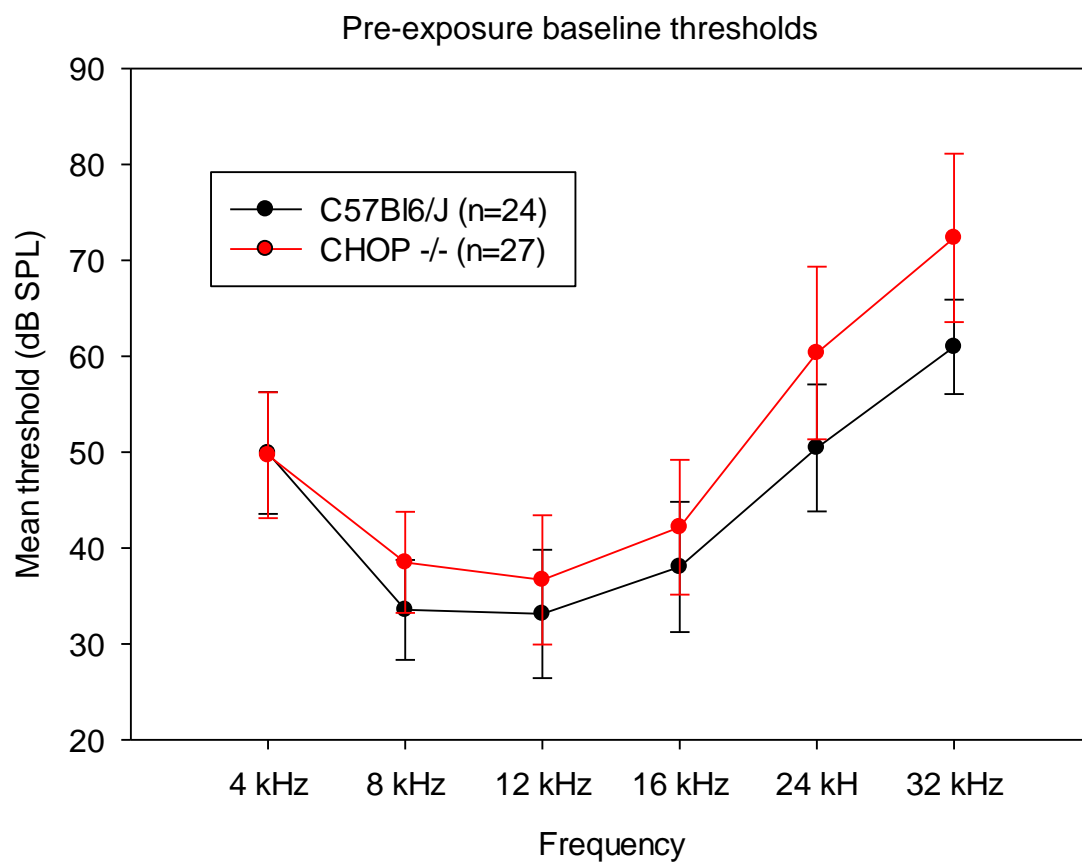


Figure 3. Pre-exposure baseline thresholds of control C57Bl6/J mice (shown in black) and CHOP -/- mice (shown in red).

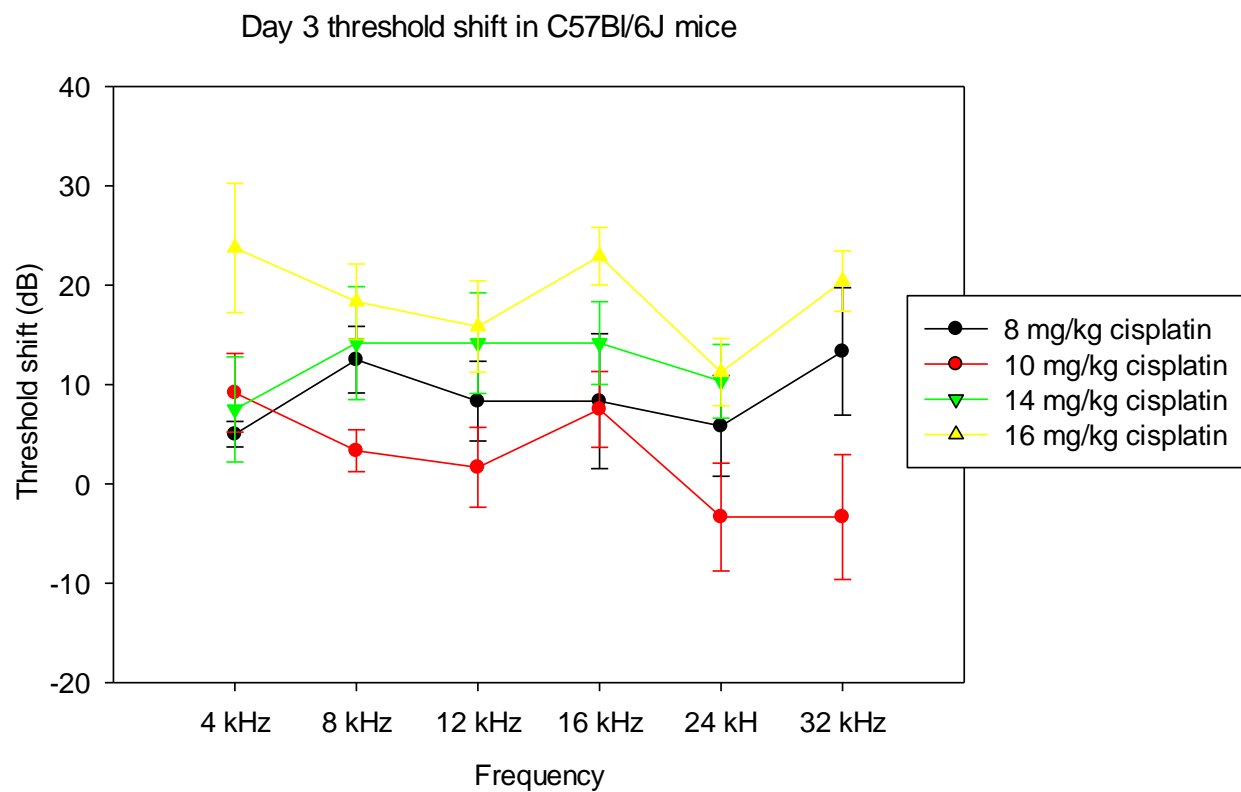


Figure 4. Threshold shift of all control C57Bl/6J mice on Day 3 post-cisplatin exposure. Different doses of cisplatin (8, 10, 14, and 16 mg/kg) are shown in different colors (black, red, green, and yellow, respectively).

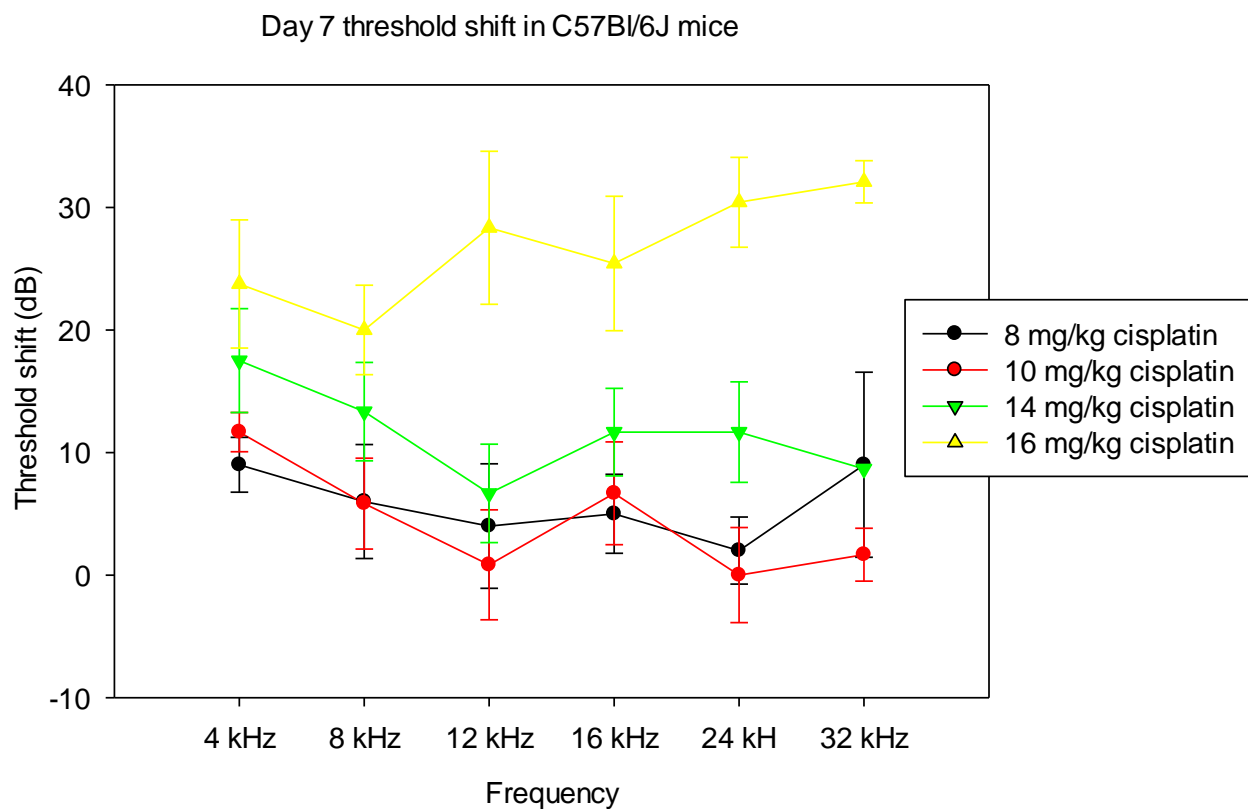


Figure 5. Threshold shift of all control C57Bl/6J mice on Day 7 post-cisplatin exposure. Different doses of cisplatin (8, 10, 14, and 16 mg/kg) are shown in different colors (black, red, green, and yellow, respectively).

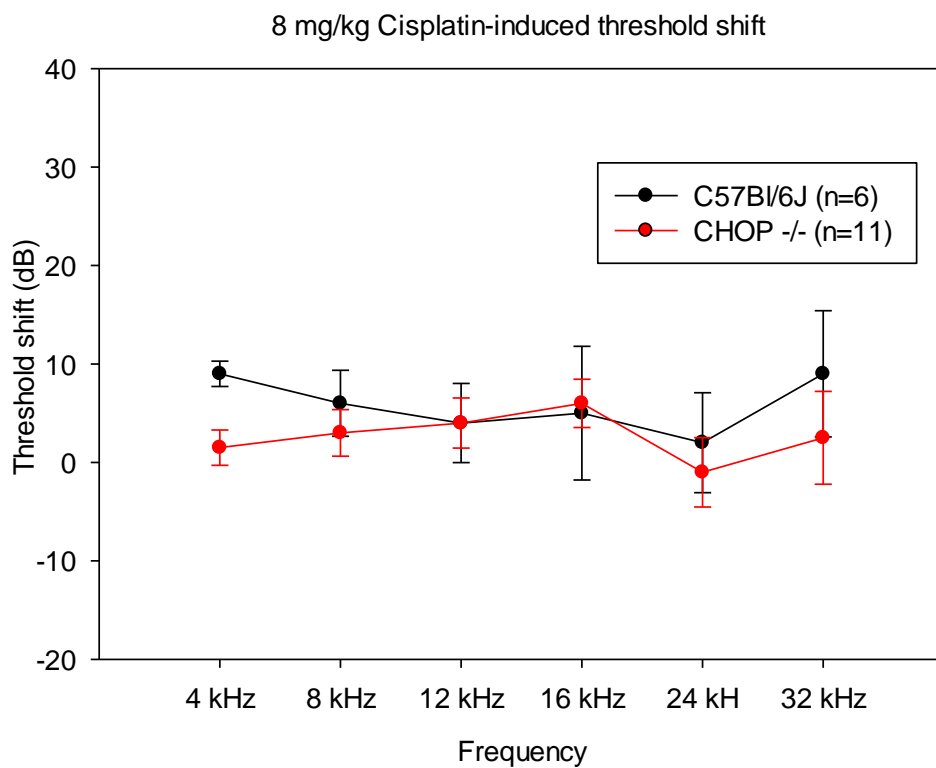


Figure 6. Threshold shift on Day 7 of mice that received the 8 mg/kg cisplatin dose. The control C57Bl/6J group is shown in black and the experimental CHOP knockout group is shown in red.

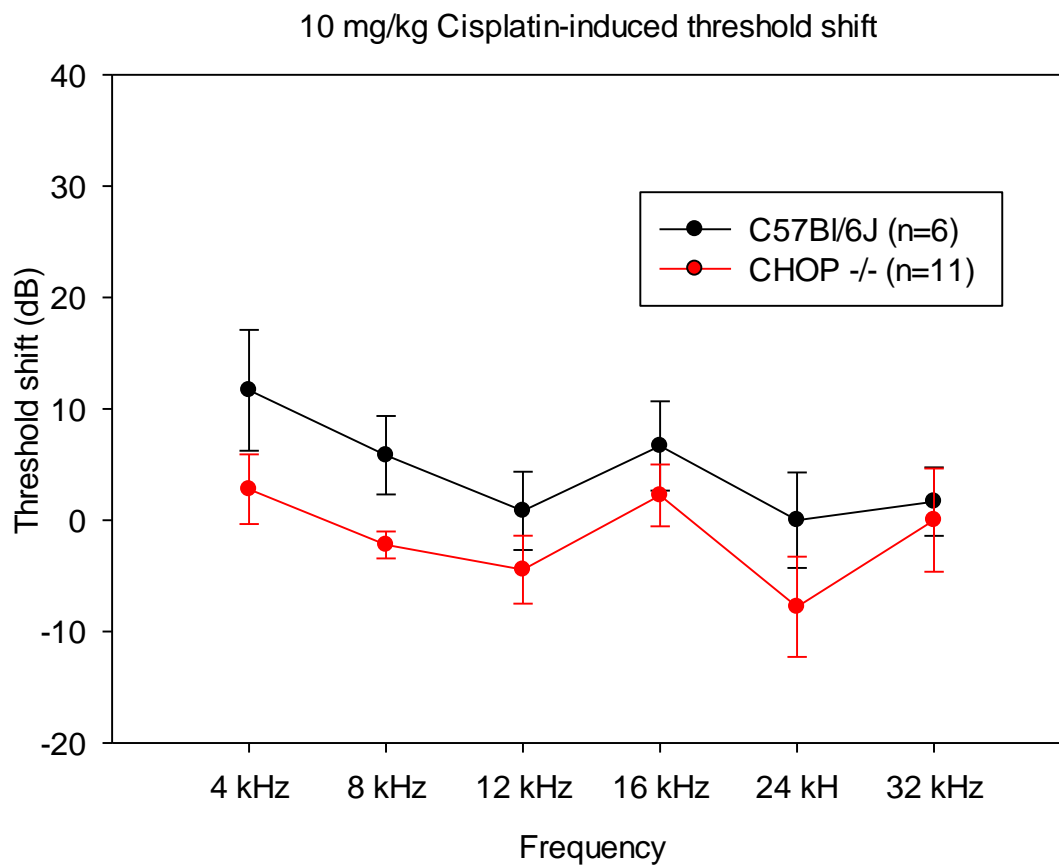


Figure 7. Threshold shift on Day 7 of mice that received the 10 mg/kg cisplatin dose. The control C57Bl/6J group is shown in black and the experimental CHOP knockout group is shown in red.

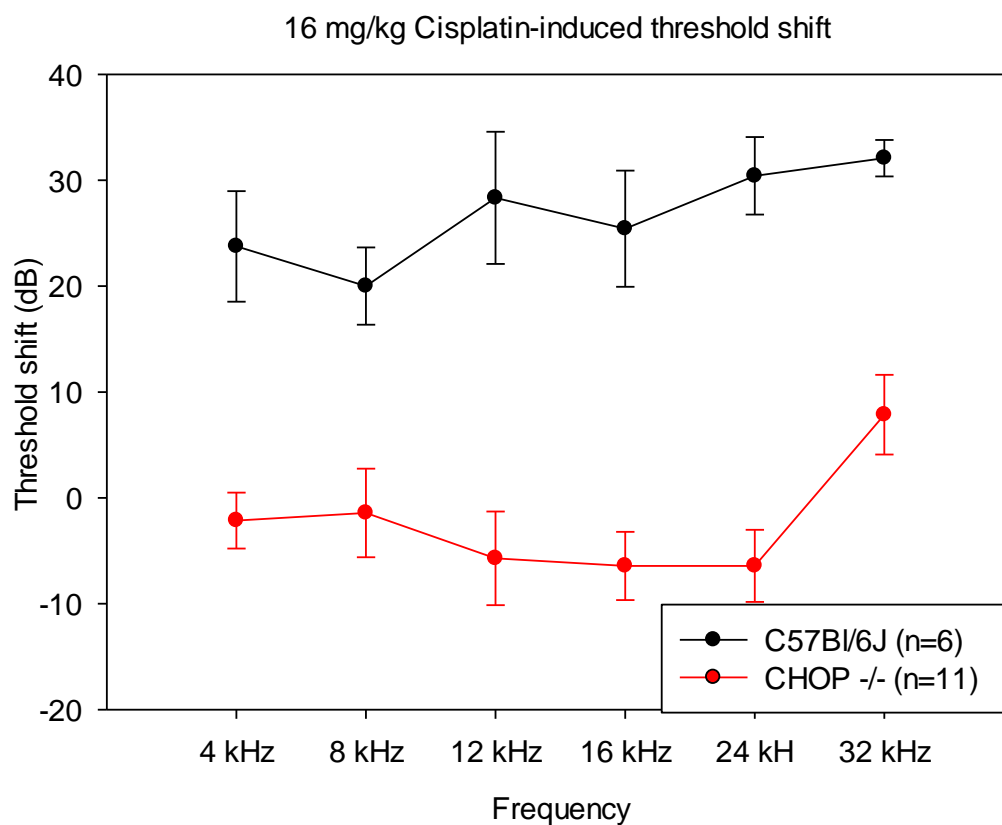


Figure 8. Threshold shift on Day 7 of mice that received the 16 mg/kg cisplatin dose. The control C57Bl/6J group is shown in black and the experimental CHOP knockout group is shown in red.

4. Discussion

In this experiment, the dose response results of cisplatin exposure in C57Bl/6J mice compared with experimental CHOP knockout mice showed a significant effect of CHOP-mediated pathways on cisplatin-induced hearing loss. At 16 mg/kg, the CHOP mice showed less than 10 dB of threshold shift, which was significantly less than the C57Bl/6J controls experienced. Increased cisplatin dosage corresponded with increased progressive hearing loss, beginning at the highest frequencies. Doses under 16 mg/kg in the C57Bl/6J controls were not progressively toxic (i.e. significant increase from Day 3 to Day 7). By Day 7 after the initial dose response testing of the control C57Bl/6J mice, the threshold shift of the three lower dose groups (8, 10, and 14 mg/kg) were not significantly different from one another (Figure 4). This was different from our expectations. Our lab's past history with cisplatin dosing has been in work with the Fischer 344 rat. The Fischer 344 rat is highly susceptible to cisplatin ototoxicity, with large threshold shifts from 12 mg/kg doses. The C57Bl/6J mouse is known to be susceptible to noise-induced and age-related hearing losses, so our expectation was that it would also be highly susceptible to cisplatin-induced hearing loss. For that reason, we began with low doses of 8 and 10 mg/kg in the controls and the CHOP $-/-$ mice. Those low doses did not create enough threshold shift in the controls to provide a good comparison to the CHOP $-/-$. Therefore, we tested 14 and 16 mg/kg doses. The threshold shift measured in the 16 mg/kg group was significant enough to be a good comparison to threshold shift in the CHOP knockout mice. As a consequence, no CHOP mice received the 14 mg/kg cisplatin dose, because we found the proper experimental dose at 16 mg/kg. The dose response threshold shift of cisplatin-induced hearing loss and greater threshold shift in Day 7 testing than Day 3 is consistent with previous research and is the result of the traveling apoptotic cochlear lesion (Fleischman et al, 1975).

An unexpected discovery was that the CHOP knockout mice did not have equivalent hearing sensitivity to the controls. They had higher baseline thresholds, indicating initial hearing loss likely due to developmental damage or malformation of the cells in the cochlea. The finding indicates that the UPR is crucial for the normal development of the auditory system. A possible explanation for this result is that cochlear development without CHOP resulted in damaged or malformed cochlear cells, indicating that CHOP-mediated UPR is a regulatory mechanism that promotes cochlear development as well as destruction (Yan et al., 2015).

The major finding from the study was less cisplatin-induced hearing loss in the CHOP knockout mice. At each drug dosage, the mean threshold shift after cisplatin was higher in the control group than in the CHOP knockout group. This supports the hypothesis that CHOP is a potential triggering molecule for cisplatin-induced apoptosis in the cochlea. The results of this project showed the involvement of CHOP-related apoptosis pathways in cisplatin-induced hearing loss, suggesting that modifications of these pathways at any point could be a target for clinical treatment of cisplatin-induced hearing loss. The development of localized gene therapy is an ambitious future goal for cisplatin-induced hearing loss. However, the incomplete protection afforded by CHOP knockout shown by the existing threshold shift in the experimental CHOP mice supports the hypothesis that there are multiple pathways for cisplatin-induced hearing loss, creating a barrier for an all-encompassing treatment. Nevertheless, if the threshold shift protection demonstrated in the current study could be applied to human cisplatin patients, it would represent a major improvement in managing ototoxicity in this patient population.

Further research is needed to more effectively model the use of cisplatin treatment in humans. This would include research paradigms with multiple doses of cisplatin over a time period to emulate human chemotherapy treatments, as well as an all-encompassing dose response

model of cisplatin-induced hearing loss in mice to compare with a human model. This would facilitate the research of better protection methods for humans. Additionally, further research would include an anatomical analysis of the cochlear hair cells in the mouse. The type and number of hair cells preserved in the CHOP mice compared to the C57Bl6/J controls would give further indication to the specificity of CHOP-mediated apoptosis pathways and targeting strategies. Those analyses are ongoing.

Ultimately, the UPR system needs to be better understood in order to promote its cytoprotective effect in the cochlea, possibly through CHOP gene manipulation. However, the cytotoxic effects of the UPR need to be retained when activated by cisplatin on the rest of the body in order to utilize cisplatin as an effective chemotherapeutic agent. In terms of future research, this study is a promising foundation to investigate UPR-mediated apoptosis pathways further, and to neutralize CHOP when ideal for its cytoprotective effect. Future studies will attempt to modulate the UPR in the cochlea as a way to prevent cisplatin ototoxicity. Additionally, the study of cisplatin-induced apoptotic pathways should be compared with similar noise-induced research in order to discover the similarities and differences between these cochlear applications.

7. References

- Ding, D., Jiang, H., Wang, P., & Salvi, R. (2007). Cell death after co-administration of cisplatin and ethacrynic acid. *Hearing Research*, 226, 129-139.
- Erguler, K., Pieri, M., & Deltas, C. (2013). A mathematical model of the unfolded protein stress response reveals the decision mechanism for recovery, adaptation and apoptosis. *BMC Systems Biology*, 7(16). Retrieved from <http://www.ebi.ac.uk/biomodels-main/static-pages.do?page=ModelMonth%2F2014-11>.
- Fleischman, R. W., Stadnicki, S. W., Ethier, M. F., & Schaeppi, U. (1975). Ototoxicity of cis-dichlorodiammine platinum (II) in the guinea pig. *Toxicology and Applied Pharmacology* 33, 320-332.
- Fujinami, Y., Mutai, H., Mizutari, K., Nakagawa, S., & Matsunaga, T. (2012). A novel animal model of hearing loss caused by acute endoplasmic reticulum stress in the cochlea. *Journal of Pharmacological Sciences* 118, 363-372.
- Hayes, D.M., Cvitkovic, E., Golbey, R.B., Scheiner, E., Helson, L., & Krakoff, I.H. (1977). High dose cis-platinum diammine dichloride: amelioration of renal toxicity by mannitol diuresis. *Cancer*, 39, 1372-1381.
- Knight, K. R. G., Kraemer, D. F., & Neuwelt, E. A. (2005). Ototoxicity in children receiving platinum chemotherapy: Underestimating a commonly occurring toxicity that may influence academic and social development. *Journal of Clinical Oncology*, 23(34), 8588-8596.
- Madasu, R., Ruckenstein, M., Leake, F., Steere, E., & Robbins, K. (1997). Ototoxic effects of supradose cisplatin with sodium thiosulfate neutralization in patients with head and neck cancer. *Archives of Otolaryngology, Head and Neck Surgery*, 123, 978-981.
- Rybak, L. P., & Ramkumar, V. (2007). Ototoxicity. *Kidney International*, 72(8), 931-935.
- Sammata, N., & McClintock, T. S. (2010). Chemical stress induces the unfolded protein response in olfactory sensory neurons. *The Journal of Comparative Neurology*, 518(10) 1825–1836.
- Wang W., Sun Y., Chen S., Zhou X., Wu X., Kong W., & Kong W. (2015). Impaired unfolded protein response in the degeneration of cochlea cells in a mouse model of age-related hearing loss. *Experimental Gerontology*, 70, 61-70.
- Yan, M., Jiangdong, N., Song, D., Ding, M., & Huang, J. (2015). Activation of unfolded protein response protects osteosarcoma cells from cisplatin-induced apoptosis through NF- κ B pathway. *International Journal of Clinical and Experimental Pathology*, 8(9), 10204-10215.
- Yost, W. A. (2008). *Fundamentals of Hearing*. (5th ed., p. 86, 235). Bingley, UK: Emerald Publishing Group.